AD_____

Award Number: DAMD17-03-1-0319

TITLE: Prion Transport to Secondary Lymphoreticular Tissues

PRINCIPAL INVESTIGATOR: Jason C. Bartz, Ph.D.

CONTRACTING ORGANIZATION: Creighton University
                                   Omaha, NE 68178

REPORT DATE: June 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
          Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                        Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-06-2008	Final	1 JUN 2003 - 31 MAY 2008

4. TITLE AND SUBTITLE

Prion Transport to Secondary Lymphoreticular Tissues

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0319

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jason C. Bartz, Ph.D.

E-Mail: jbartz@creighton.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Creighton University
Omaha, NE 68178

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The long-term objective of this proposal is to identify mechanisms of prion transport to secondary lymphoreticular system (LRS) tissues. The hypothesis to be tested is that following peripheral exposure to prions; host proteins (e.g. complement) bind prions allowing for trapping by cells in the spleen and enhancing uptake by macrophages, which are cells that are responsible for destruction of foreign proteins. To investigate this hypothesis we will examine the disease development of a prion strain (DY TME) that does not replicate in the spleen of hamsters. This system will provide details into the host factor(s) involved in transport of prions to cells in the LRS. We have shown the susceptibility of HY and DY TME to phagocytosis and degradation by a murine macrophage cell line. We have studied the effects of prion infection on phagocytic ability and cell viability. We have shown differences in the spatial and temporal spread of the HY and DY TME agents in LRS tissues following intraperitoneal inoculation. We have shown gender specific responses to intraperitoneal DY TME inoculation.

15. SUBJECT TERMS
Prion diseases, macrophage, complement

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	15	**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction

The long-term objective of this proposal is to identify mechanisms of prion transport to secondary lymphoreticular system (LRS) tissues. The hypothesis to be tested is that following peripheral exposure to prions; host proteins (e.g. complement) bind prions allowing for trapping by cells in the spleen and enhancing uptake by macrophages, which are cells that are responsible for destruction of foreign proteins. To investigate this hypothesis two animal models will be used. Genetically engineered mice that lack components of the complement system will be used to test the hypothesis that complement binding to PrP^{Sc} is involved in targeting of prions to cells in the spleen and uptake by macrophages. A second system will examine disease development of a prion strain (DY TME) that does not replicate in the spleen of hamsters. We will use this system to test the hypothesis that DY TME is not bound by complement resulting in its absence in the spleen. The mouse and hamster systems investigate prion interactions with complement components based on differences of host and strain properties, respectively. This study will provide details into the host factor(s) involved in transport of prions to cells in the LRS, such as spleen.

Body

Tissue distribution of HY & DY TME at early time points post-infection. As previously reported, animal bioassay was used to investigate if DY PrP^{Sc} is present in spleen, lymph nodes, and peritoneal cells following intraperitoneal inoculation with DY TME as outlined in task 4. Hamsters were intraperitoneally (i.p.) inoculated with DY TME and spleen, mesenteric lymph node, medial iliac lymph node and peritoneal cells (i.e. peritoneal lavage) were collected from two hamsters at both 60 and 120 days post DY TME i.p. infection. As a negative control, the same tissues were also collected from mock-infected hamsters. The hamsters inoculated with the tissue collected at 60 and 120 days from the DY TME infected hamsters did not result in clinical symptoms of DY TME by 400 days post-infection when the experiment was terminated (Bartz, 2004). Similarly, the tissues collected from the mock-infected hamsters did not cause disease by 400 days post-infection when the experiment was terminated. These data indicate that DY TME is not transported to or does not replicate in spleen and lymph node.

Next, we investigated the distribution of infectivity and PrP^{Sc} in hamsters infected with the HY or DY TME agents at early time points post-infection. Hamsters were intraperitoneally inoculated with $10^{5.5}$ LD$_{50}$ of DY TME or $10^{8.5}$ LD$_{50}$ of HY TME. At 1, 2, 4, 8, 16, 32, 64 hours, 1, 2, 4, 6, 8, and 11 weeks post-infection, three animals for each inoculation group were sacrificed and peritoneal cells, spleen, mesenteric lymph node, medial iliac lymph node, and submandibular lymph node were collected. We used detergent extraction and ultracentrifugation to enrich for PrP^{Sc} prior to Western blot analysis. HY PrP^{Sc} is present in the peritoneal cell collections from 1 hour post-infection through 2 weeks post-infection. HY PrP^{Sc} is present in the medial iliac lymph node and

4

spleen from 1 hour post-infection to 32 hours post-infection. HY PrP^{Sc} is present in the mesenteric lymph node by 2 hours post-infection, and remains in this tissue through 32 hours post-infection (Bartz, 2007). In contrast, DY PrP^{Sc} is present in the peritoneal cells from 1 hour post-infection through 32 hours post-infection. DY PrP^{Sc} is detectable in the medial iliac lymph node from 2 hours post-infection to 32 hours post-infection. DY PrP^{Sc} is undetectable in the mesenteric lymph node at all time points, and is detectable in the spleen between 1 and 4 hours post-infection (Bartz, 2007). These data indicate that both HY and DY TME agents are transported to secondary lymphoreticular system tissues following intraperitoneal inoculation. The inability of DY TME to cause disease following intraperitoneal inoculation does not appear to be related to a lack of transport of the agent from the periphery to the draining lymph nodes. DY TME is transported to the medial iliac lymph node, the local, draining lymph node of the peritoneal cavity, in a time frame similar to HY TME.

Complement levels in TME-infected brain and spleen. To begin to investigate the interaction of complement with PrP^{Sc} as outlined in task 5, Western blot analysis was used to measure the abundance of C1q in prion-infected brain and spleen homogenates and in preparations enriched for HY or DY PrP^{Sc}. Western blot analysis was performed on spleen and brain homogenates from HY TME, DY TME or mock-infected hamsters for the presence of C1q using a polyclonal anti-human C1q antibody. The abundance of C1q was similar between HY TME, DY TME and mock-infected brain homogenates, and HY TME and mock-infected spleen homogenates (Bartz, 2004).

To investigate if C1q is directly bound to PrP^{Sc}, PrP^{Sc} enriched preparations from HY and DY TME-infected brains were prepared by detergent extraction and differential centrifugation as previously described (Bartz et al., 2004). The PrP^{Sc} enriched preparations were analyzed for the presence of C1q by Western blot analysis with the polyclonal anti-human C1q antibody. C1q was not detected in PrP^{Sc}-enriched preparations from HY or DY TME-infected brain tissue (data not shown).

Previously we reported we that HY and DY PrP^{Sc} can selectively be immunoprecipitated from brain tissue (Bartz, 2005). We used this technique to immunoprecipitate PrP^{Sc} and perform Western blot analysis for molecules that we hypothesized would bind to PrP^{Sc} (e.g. C1q). To further investigate if C1q is directly bound to PrP^{Sc} we adapted the immunoprecipitation protocol using the 15B3 antibody. For immunoprecipitation of PrP^{Sc}, M-450 rat anti-mouse IgM dynabeads (Dynal biotech, Oslo, Norway) are prepared for immunoprecipitation according to manufacturer's instructions and are incubated with the 15B3 antibody (a generous gift from Alex Rabier and Bruno Oesch, Prionics AG, Switzerland; Nazor et al., 2005) for 2 hours at room temperature with gentle shaking. After incubation, the beads are placed on a magnetic particle concentrator (MPC-S; Dynal Biotech, Oslo, Norway) and the supernatant containing unbound 15B3 is removed and discarded. The beads are washed by the addition of five volumes of coating buffer [0.1% v/w BSA (Sigma-Aldrich, St. Louis, MO) in DPBS (Mediatech, Inc., Herdon, VA)] followed by vortexing. The

beads are concentrated using the MPC-S and the supernatant is removed. After three washes, the beads are resuspended in one volume of coating buffer and stored at 4°C. To 30 μl of the prepared beads, 250 μg brain equivalents of brain homogenate containing protease inhibitors (complete protease inhibitors – Roche Diagnostics GmbH, Manheim, Germany) and 445 μl of immunoprecipitation buffer (0.3% sarkosyl in TBS) are added and incubated at room temperature with gentle shaking for two hours. The 15B3 coated magnetic beads that has bound PrP^{Sc} are collected with the MPC-S magnet and the supernatant containing PrP^{C} is removed. After addition of 1ml wash buffer, PrP^{Sc} bound to the magnetic beads via 15B3 is collected using the MPC-S magnet. The supernatant is removed and discarded. The PrP^{Sc} is separated from the beads by boiling in 25 μl of SDS-PAGE loading buffer for five minutes. The beads are collected with the MPC-S magnet and the supernatant containing PrP^{Sc} is removed. The binding of PrP^{Sc} and C1q is analyzed by Western blot using the anti-C1q antibody. We were unable to detect C1q bound to the immunoprecipitated PrP^{Sc} via this method (data not shown).

These findings suggest that C1q levels are not increased in brain tissue of TME-infected hamsters, and is consistent with the observation that i.c. inoculation of complement deficient mice has the same incubation period as wild type mice (Klein et al., 2001; Mabbott et al., 2001). These data are consistent with the hypothesis that complement does not contribute to pathogenesis in the central nervous system. The similar abundance of C1q in HY TME-infected spleen compared to mock-infected hamsters suggests that C1q is not involved in HY TME replication in the spleen and suggests that the increase in incubation period in complement deficient mice inoculated i.p. is due to prion opsonization and transport to the spleen.

Antibody response to DY PrP^{Sc}. Recent reports have suggested that antibodies directed against PrP can increase the incubation period of prion disease (Schwarz et al., 2003; Sigurdsson et al., 2003; Beringue et al., 2004). This suggested to us that a possible mechanism for the ability of DY TME to extend the incubation period of superinfected HY TME following i.p. inoculation was due to generation of antibodies against DY PrP^{Sc} that would cross react with HY PrP^{Sc}. To investigate if antibodies are generated against DY PrP^{Sc}, we developed a 96 well immunoassay to detect PrP specific antibodies as previously reported (Bartz, 2004). Using this method we have plated brain tissue homogenized in the presence of protease inhibitors or proteinase K digested from at least 3 individual uninfected, DY TME and HY TME-infected hamsters. Using these tissues as the "antigen" we have investigated for the presence of antibodies in serum from 6 uninfected animals as a negative control and at least three individual hamsters infected with DY TME by either the intracerebral, intraperitoneal or intralymph node routes of infection. We also investigated for the presence of antibodies in serum from three hamsters intracerebrally infected with HY TME. Using these combinations of brain tissue and serum we were unable to identify an antibody response in HY TME or DY TME-infected hamsters. This is a significant finding and excludes the possibility that the

extension of superinfected HY TME is due to an antibody response to PrP or that antibody opsonization of PrP^{Sc} facilitates prion transport.

Serum amyloid protein (i.e. Female protein) levels in prion-infected hamsters. It is possible that the observed increase in incubation period in complement deficient animals is not due to a direct interaction of complement components with PrP^{Sc} but via an intermediate molecule. A possible candidate molecule is serum amyloid protein (SAP) that has been shown to bind to amyloid and can also directly bind to C1q (Coe and Ross, 1990; Nauta et. al., 2003). Uninfected, HY or DY TME-infected brain and spleen homogenates were size fractioned on 12.5% SDS-PAGE and transferred to Immobilon P as previously described (Bartz et al., 2004). The Western blot was incubated with rabbit anti-female protein, the hamster homolog of SAP. Our experiments have indicated that while there are no differences in migration or abundance of FP in uninfected and HY TME-infected spleen, a difference in the banding pattern of FP was observed in HY TME-infected brain compared to an uninfected control (Bartz, 2005).

Response of female hamsters to DY TME-infection. In hamsters, female protein is regulated by estrogen resulting in approximately 100-fold higher serum level of female protein compared to male hamsters (Coe & Ross, 1990). To investigate if this higher level of serum protein had an effect on DY TME incubation period and DY PrP^{Sc} properties, male and female hamsters were intracerebrally inoculated with $10^{3.8}$ LD$_{50}$ of DY TME. As previously reported, the incubation period of DY TME in male and female hamsters was not statistically significant (p>0.05 Student's T-test; Bartz, 2005). This differs from previous studies that indicated an eight percent reduction in the incubation period of hamsters i.c. inoculated with the 263K strain of hamster-adapted scrapie (Kimberlin & Walker, 1977) but is consistent with studies in mice where the incubation period in females is not uniformly shorter than in males (Outram, 1976).

Following intraperitoneal inoculation of male hamsters with the DY TME agent derived from males, the animals do not express clinical symptoms and DY PrP^{Sc} is undetectable in secondary lymphoreticular tissues and brain (Bartz et al., 2005). To investigate if higher levels of serum protein had an effect on the ability of DY TME to cause disease following intraperitoneal inoculation, $10^{4.5}$ LD$_{50}$ of DY TME from male hamsters was intraperitoneally inoculated into female recipient hamsters. One out of the four female hamsters that was intraperitoneally inoculated exhibited DY TME clinical symptoms at 224 days post-inoculation. DY PrP^{Sc} was present in the brain at the time of sacrifice. This is the first known case of DY TME causing disease following a non-neuronal route of inoculation. Following enrichment for PrP^{Sc} by detergent extraction and ultracentrifugation, none of the secondary lymphoreticular tissues tested contained detectable levels of DY PrP^{Sc} (Bartz, 2006).

To further investigate this result, we repeated the experiment using DY TME derived from male and female hamsters. As previously reported, one out of the six male hamsters inoculated with male DY TME exhibited clinical symptoms, and one out of the six female hamsters inoculated with male DY TME exhibited

clinical symptoms (Bartz, 2007). Four out of the six male hamsters inoculated with female DY TME exhibited clinical symptoms and one out of the six female hamsters that was inoculated with female DY TME exhibited clinical symptoms. DY PrP^{Sc} was present in the brains of each of these animals at the time of sacrifice (Bartz, 2007), and the brain homogenate of an additional male hamster inoculated with female DY TME was found PrP^{Sc} positive by Western blot analysis (data not shown).

Macrophage degradation of TME PrP^{Sc}. The interactions of HY and DY TME PrP^{Sc} and macrophages were investigated as outlined in task 6. Murine macrophage RAW 264.7 cells were grown to confluence in DMEM (ATCC, Manassas, VA) containing 10% fetal bovine serum (ATCC, Manassas, VA) and 100 U/ml of penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO). The cells were counted using a hemocytometer (Hausser Scientific, Horsham, PA) and the cell concentration was adjusted to 10^6 cells per ml using pre-warmed (37°C) DMEM Media. A total of 10^5 cells per well were placed in a plastic 96-well cell culture plate. The cells were allowed to adhere to the plate for 1 hour.

      To assess if RAW 264.7 cells could degrade HY or DY PrP^{Sc}, PrP^{Sc} was incubated with either 10^5 RAW 264.7 cells or an equal volume of DMEM Media without cells as a control for non-macrophage mediated PrP^{Sc} degradation. HY and DY TME brain homogenates were prepared in PBS and were not digested with proteinase K. 250 µg equivalents of brain homogenate in DMEM Media were added to each well. Samples in triplicate were collected at 1, 2, 4, 6, 12, 18, 24, 48, and 72 hours post-TME-infection. At each collection point the media was removed and saved. An equal volume of DMEM Media was added to the well to collect the remaining free PrP^{Sc} and was added to the previously collected media. The PrP^{Sc} in the media and wash is referred to as the media associated PrP. To the cells, 100µl of 0.1% w/v NLS was added to the well to dislodge the cells from the well and collected. The wells were then washed with 100 µl of 0.1% w/v NLS that was added to the first cell collection. The PrP^{Sc} collected in 0.1% NLS is referred to as cell-associated PrP. The levels of PrP in the media and associated with the cells were quantified using Western blot analysis with the anti-PrP monoclonal antibody, 3F4.

      The murine macrophage cell line RAW 264.7 cells degrade both HY and DY PrP^{Sc}. Cell-associated PrP^{Sc} levels peaked by 24 hours, and were significantly degraded by 72 hours (Bartz, 2007). This suggests that both HY and DY TME may be phagocytosed and degraded by lymphatic tissue macrophages. Although our initial experiments with primary hamster adherent peritoneal cells indicated a differential degradation of HY and DY TME, these data suggest that the two strains are processed by murine macrophages in a similar manner. The inability of DY TME to cause disease following intraperitoneal inoculation does not appear to be related to faster uptake and/or degradation of the agent.

Cell viability: Following the co-culture protocol above, RAW 264.7 cells were cultured with HY or DY TME infected brain homogenate. Following 72 hours of co-culture, the cells were assessed for viability using the CellTiter-Glo Cell Viability Assay according to manufacturer's directions (Promega, Madison, WI). Briefly, the supernatant is removed from the cells, and they are incubated with CellTiter-Glo Buffer for 1 hour at 37°C. The culture is then equilibrated to room temperature for 30 minutes and Cell Viability Substrate is added to each well. Cell lysis is induced by mixing the contents on an orbital shaker for 2 minutes. The culture is incubated for 10 minutes to stabilize the luminescence. The chemiluminescence signal is detected using a Kodak 2000R imaging station (Kodak, Rochester, NY) and is directly proportional to the amount of ATP released from the cells during lysis. We observed an increase in the number of viable cells during the 0 to 72 hours of culture (Figure 1). This result indicates that the cell line is undergoing cellular division during the time frame of the experiment. When the cells are co-cultured with either HY or DY TME infected brain homogenate, we again observed an increase in the number of viable cells, and there is no significant
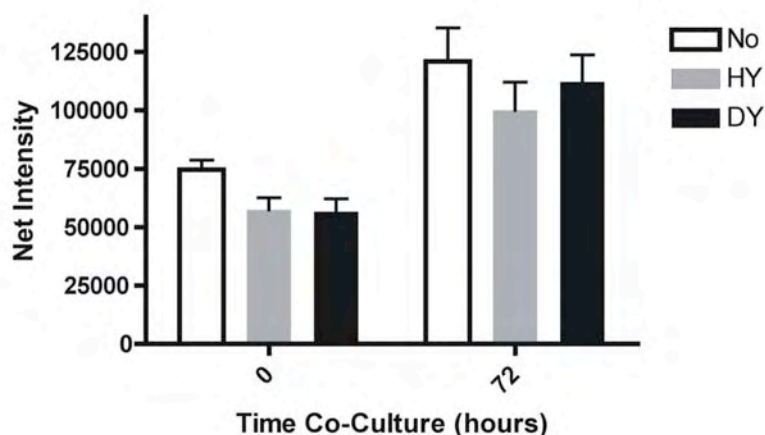

Figure 1. CellTiter-Glo cell viability of RAW 264.7 cells at 0 and 72 hours post co-culture with no brain homogenate, HY, or DY TME infected brain homogenate.

difference between the number of viable cells in the non-infected, HY, or DY TME infected groups (Figure 1). These data indicate that co-culture of RAW 264.7 cells with HY or DY TME infected brain homogenate does not adversely affect cellular viability.

Macrophage phagocytosis: Following the co-culture protocol above, RAW 264.7 cells were cultured with HY or DY TME infected brain homogenate. After 72 hours of co-culture, normal macrophage function was assessed by measuring the ability of the cells to phagocytose fluorescent bioparticles. This assessment was done with the Vybrant Phagocytosis Assay Kit (Molecular Probes, Eugene, OR) per the manufacturer's instructions. Briefly, the supernatant is removed from the cells and 100 µl of fluorescent bioparticle solution is added to each well. Following a 2 hour incubation at 37°C, the remaining bioparticle solution is removed and trypan blue is added to each well for 1 minute to quench the extracellular fluorescence. The culture is then examined under fluorescence
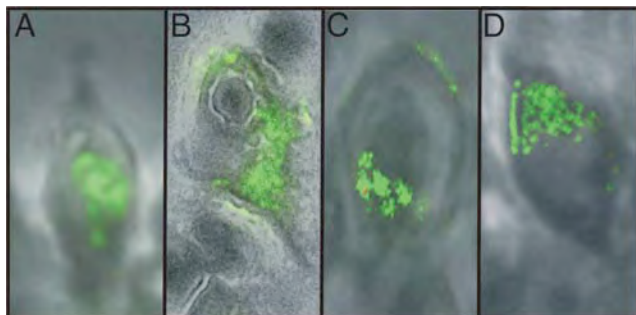
Figure 2. Vybrant Phagocytosis Assay of RAW 264.7 cells at 0 and 72 hours post co-culture with no brain homogenate, HY, or DY TME infected brain homogenate. A) Cell at 0 hours, no brain homogenate; B) Cell at 72 hours, no brain homogenate; C) Cell at 72 hours, HY co-culture; D) Cell at 72 hours, DY co-culture.

microscopy using a Nikon eclipse TS100 microscope (Melville, NY) and imaged with a DigiFire camera and analysis software (Soft Imaging System, Lakewood, CO). We observed intracellular fluorescence at 0 and 72 hours of culture (Figure 2, Panels A and B, respectively). These data indicate that the cell line is capable of phagocytosis during the time frame of the experiment. When the cells are co-cultured with either HY or DY TME infected brain homogenate, we again observed intracellular fluorescence (Figure 2, Panels C and D, respectively). These data indicate that co-culture of RAW 264.7 cells with HY or DY TME infected brain homogenate does not abolish cellular phagocytosis.

Replication of HY and DY TME agents in lymphoid tissue. It is possible that the reduced ability of DY TME to cause disease following peripheral routes of infection is due to a lack of agent replication within the secondary lymphoreticular system tissues. To investigate this possibility, we have been studying agent replication using an adapted form of the protein misfolding cyclic amplification (PMCA) protocol (Castilla et al., 2005). Uninfected, HY, and DY TME infected hamsters were perfused with phosphate buffered saline (PBS) containing 5 mM EDTA. Ten percent w/v brain and spleen homogenates were prepared in conversion buffer (PBS containing 150 mM NaCl, 1.0% Triton X-100, 4 mM EDTA, and complete protease inhibitor cocktail (Boehinger Mannheim, Mannheim, Germany)) from each animal. Clarified brain homogenate was produced by low-speed centrifugation (500 x g for 30 seconds). Ninety-five μl aliquots of the 10% w/v uninfected brain or spleen homogenate was mixed with 5 μl of the 10% w/v uninfected, HY, or DY TME infected brain homogenate in PCR tubes. 15 μl aliquots were transferred from each tube to a fresh PCR tube and frozen at -80°C for comparison of initial PrP^{Sc} content. The tubes for PMCA were placed in the cuphorn microsonicator (Misonix Model 3000, Farmingdale, NY). The samples underwent cycles consisting of 10 minutes of incubation in a 37°C water bath followed by 5 seconds of sonication with the apparatus set at 60% power. The samples were removed after two hours, centrifuged for 5 seconds and frozen at -80°C. Each sample was treated with proteinase K digestion and size-fractioned on 12.5% SDS-PAGE gel as previously described (Bartz et al., 2007). PrP^{Sc} content of the frozen and sonicated samples was determined by Western blot analysis using the anti-prion protein antibody 3F4. During the 2 hours of incubation and sonication cycles, there was no amplification of PrP^{Sc} in
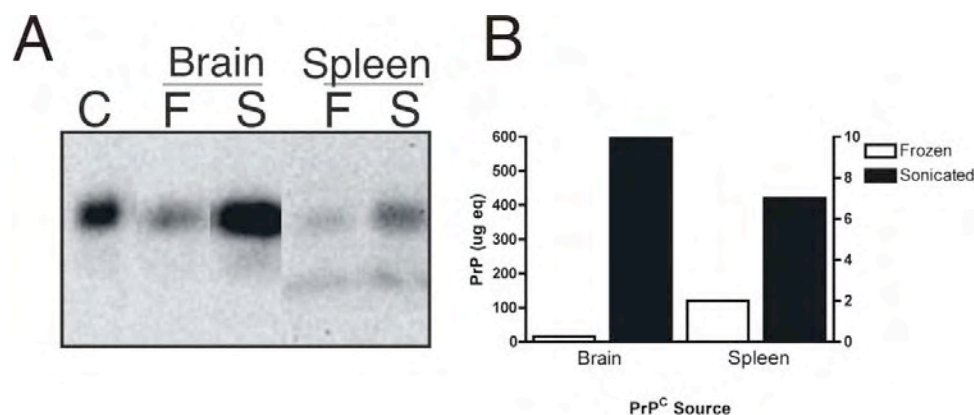
Figure 3. A) Western blot analysis of brain and spleen homogenate from frozen and PMCA sonicated samples. (C-25 μg eq HY-infected brain homogenate; F-initial frozen collection, 250 μg eq; S-sonicated collection, 250 μg eq) B) Quantification of PrP^{Sc} (μg eq) from Western blot analysis using GraphPad Prism Software.

our uninfected controls (data not shown). We observed a 40-fold increase in the amount of HY PrP^{Sc} amplified in brain homogenate and a 3.5-fold increase in the amount of HY PrP^{Sc} amplified in spleen homogenate (Figure 3). This is the first report of prion amplification in lymphoid system tissues using PMCA. During our initial studies, we were unable to amplify DY PrP^{Sc} in spleen homogenate. Our current studies are focused on modifying the PMCA protocol to determine if increased amounts of DY PrP^{Sc} in the mixture or additional cycles of incubation and sonication will allow for conversion in spleen homogenate.

Key Research Accomplishments

1. Demonstrated that spleen, mesenteric lymph node, medial iliac lymph node, and peritoneal cells from hamsters i.p. inoculated with DY TME at 60 or 120 days post infection have no detectable DY TME infectivity when tested by animal bioassay.
2. Peritoneal cells and lymphoreticular system tissues from uninfected, HY TME and DY TME-infected hamsters have been collected and we determined the spatial and temporal spread of PrP^{Sc} in these tissues.
3. C1q abundance in spleen and brain from HY and DY TME-infected hamsters is not altered compared to mock-infected hamsters as by determined by Western blot analysis.
4. Demonstrated though Western blot analysis of PrP^{Sc}-enriched preparations of brain homogenate and immunoprecipitation that direct binding of PrP^{Sc} and C1q are undetectable.
5. Confirmed the lack of detectable antibody response to prion infection.
6. Western blot analysis of female protein in brain demonstrates differences in banding pattern in HY TME-infected hamsters compared to uninfected control animals

11

7. Determined that female hamsters are susceptible to intracerebral DY TME infection with the same incubation period as male hamsters.
8. Gender specific specificity to DY TME intraperitoneal infection has been determined.
9. Co-culture experiments of murine macrophage RAW 264.7 cell line and PrP^{Sc} from HY and DY TME-infected hamsters demonstrated PrP^{Sc} phagocytosis and degradation of the infectious agents.
10. Effects of prion infection on macrophages were determined via cell viability and phagocytosis assays.
11. Demonstrated ability to amplify HY TME in brain and spleen homogenate using protein misfolding cyclic amplification.

Reportable Outcomes

Published Abstracts

Kramer, M.L., and Bartz, J.C. (2005). Prion Transport to Secondary Lymphoreticular System Tissues. The Second International Chronic Wasting Disease Symposium, Madison, WI. July 12-14, 2005.

Kramer, M.L., Houseman, R.C., and Bartz, J.C. (2005). Prion Opsonization and Extraneural Transport. Nebraska Center for Virology Annual Symposium, Lincoln, NE. October 7th, 2005.

Kramer, M.L., and Bartz, J.C. (2005). Prion Transport to Secondary Lymphoreticular System Tissues. Fall 2005 National Prion Research Program Investigators Meeting, Chantilly, VA. December 8-9, 2005.

Kramer, M.L., Houseman, R.C., Estrada, E.J., and Bartz, J.C. (2006). Prion Opsonization and Extraneural Transport. The 37th Annual Midwest Student Biomedical Research Forum, Omaha, NE. February 17-18, 2006.

Kramer, M.L., Houseman, R.C., Estrada, E.J., and Bartz, J.C. (2006). Prion Opsonization and Extraneural Transport. Nebraska Center for Virology Annual Flyswat Meeting, Nebraska City, NE. March 12-13, 2006.

Kramer, M.L., and Bartz, J.C. (2007). Extraneural Transport of the Prion Protein. The 38th Annual Midwest Student Biomedical Research Forum, Omaha, NE. February 23-24, 2007.

Kramer, M.L., and Bartz, J.C. (2007). Extraneural Transport of the Prion Protein. Nebraska Center for Virology Annual Flyswat Meeting, Nebraska City, NE. March 11-12, 2007.

Kramer, M.L., and Bartz, J.C. (2007). Extraneural Transport and Clearance of the Prion Protein. Keystone Symposium, The Macrophage: Homeostasis, Immunoregulation and Disease, Copper Mountain, CO. April 11-16, 2007.

Kramer, M.L., and Bartz, J.C. (2007). Transport and Clearance of Hamster Adapted Transmissible Mink Encephalopathy following Intraperitoneal Inoculation. Neuroprion 2007, Edinburgh, Scotland. September 25-28, 2007.

Kramer, M.L., and Bartz, J.C. (2007). Transport and Clearance of Hamster Adapted Transmissible Mink Encephalopathy following Intraperitoneal

Inoculation. Nebraska Center for Virology Annual Symposium, Lincoln, NE. October 26, 2007.

Kramer, M.L., and Bartz, J.C. (2008). Macrophage Uptake and Degradation of the Prion Protein. The 39th Annual Midwest Student Biomedical Research Forum, Omaha, NE. February 29-March 1, 2008.

Kramer, M.L., and Bartz, J.C. (2008). Macrophage Uptake and Degradation of the Prion Protein. Nebraska Center for Virology Annual Flyswat Meeting, Nebraska City, NE. March 16-17, 2008.

Personnel receiving pay from research effort

Bartz, Jason C. – Primary Investigator
Kramer, Michelle L. – Research Tec, Grad Student
Estrada, Elizabeth J. – Research Tec
Rhatigan, Rachel L. – Research Tec
Ayers, Jacob I. – Grad Student
Feilmann, Ian B. – Research Tec
Sheehan, Meghan H. – Research Tec

Conclusions

At 60 and 120 days post intraperitoneal infection with DY TME, the DY TME agent is not present in the spleen, mesenteric lymph node, medial iliac lymph node, and peritoneal cells as determined by animal bioassay. This data is consistent with the hypothesis that DY TME is a lymphoreticular system replication deficient prion strain. We have collected peritoneal cells and lymphoreticular system tissues from hamsters intraperitoneally inoculated with HY or DY TME and have of analyzed these tissues for the presence of PrP^{Sc} through 11 weeks post-infection. We have shown that both agents are transported to spleen and lymph nodes during the same time frame.

We have shown that HY and DY PrP^{Sc} can selectively be immunoprecipitated from brain tissue. We have used this technique to immunoprecipitate PrP^{Sc} and perform Western blot analysis for molecules that we hypothesize bind to PrP^{Sc} (e.g. C1q). We were unable to detect direct binding of C1q via this method, and these results were confirmed by Western blot analysis of C1q from PrP^{Sc}-enriched preparations. The levels of C1q are unchanged in brain and spleen homogenate of hamsters infected with HY and DY TME compared to mock infected controls. It is possible that the observed increase in incubation period in complement deficient animals is not due to a direct interaction between complement and PrP^{Sc} but could be mediated by an intermediate molecule.

We have investigated the abundance of female protein in hamsters, the hamster homolog of serum amyloid protein, and have evidence that in HY TME-infected animals female protein expression is altered. We have demonstrated that female hamsters, that express higher levels of female protein than male hamsters, are susceptible to intracerebral inoculation with DY TME and the

13

incubation period is not different than male hamsters intracerebrally inoculated with the same dose of DY TME. We have also demonstrated that female hamsters are more highly susceptible to intraperitoneal inoculation with DY TME than male hamsters intraperitoneally inoculated with the same dose of DY TME.

      We have shown phagocytosis and degradation of HY and DY PrP^{Sc} by the murine macrophage cell line RAW 264.7 cells. We have shown that following co-culture, the number of viable cells and their ability to undergo phagocytosis is unchanged compared to uninfected controls.

      Finally, we have begun studies on prion replication in lymphatic system tissues. We have shown HY PrP^{Sc} amplifies in brain and spleen homogenate using protein misfolding cyclic amplification, indicating replication of the infectious agent. We are using this protocol to determine if we can detect amplification, or replication, of DY PrP^{Sc} within spleen tissue.

References

Bartz, J. C. (2004). Prion transport to secondary lymphoreticular tissues. DOD Annual Report for June 1, 2003-May 31, 2004 on award DAMD17-03-1-0319.

Bartz, J. C., Aiken, J. M. & Bessen, R. A. (2004). Delay in onset of prion disease for the HY strain of transmissible mink encephalopathy as a result of prior peripheral inoculation with the replication-deficient DY strain. *J Gen Virol* **85**, 265-73.

Bartz, J. C. (2005). Prion transport to secondary lymphoreticular tissues. DOD Annual Report for June 1, 2004-May 31, 2005 on award DAMD17-03-1-0319.

Bartz, J. C., DeJoia, C., Tucker, T., Kincaid, A. E., & Bessen, R. A. (2005). Extraneural prion neuroinvasion without lymphoreticular system infection. *J. Virol.* **79**, 11858-11863.

Bartz, J. C. (2006). Prion transport to secondary lymphoreticular tissues. DOD Annual Report for June 1, 2005-May 31, 2006 on award DAMD17-03-1-0319.

Bartz, J. C. (2007). Prion transport to secondary lymphoreticular tissues. DOD Annual Report for June 1, 2006-May 31, 2007 on award DAMD17-03-1-0319.

Bartz, J. C., Kramer, M. L., Sheehan, M. H., Hutter, J. A. L., Ayers, J. I., Bessen, R. A., & Kincaid, A. E. (2007). Prion Interference is due to a reduction in strain-specific PrP^{Sc} Levels. *J. Virol.* **81**, 689-697.

Beringue, V., Vilette, D., Mallinson, G., Archer, F., Kaisa, M., Tayebi, M., Jackson, G.S., Clarke, A.R., Laude, H., Collinge, J. & Hawke, S. (2004). PrP^{Sc} binding antibodies are potent inhibitors of prion replication in cell lines. *J. Biol. Chem.* **279**, 39671-39676.

Castilla, J., Saa, P., Hetz, C., Soto, C. (2005). In Vitro Generation of Infectious Scrapie Prions. *Cell.* **121**, 195-206.

Coe, J.E. & Ross, M.J. (1990). Amyloidosis and female protein in the Syrian hamster. *J. Exp. Med.* **171**, 1257-1267.

Kimberlin, R.H. & Walker, C.A. (1977). Characteristics of a short incubation model of scrapie in the golden hamster. *J. Gen. Virol.* **34**, 295-304.

Klein MA, Kaeser PS, Schwarz P, Weyd H, Xenarios I, Zinkernagel RM, Carroll MC, Verbeek JS, Botto M, Walport MJ, Molina H, Kalinke U, Acha-Orbea H & Aguzzi A. (2001). Complement facilitates early prion pathogenesis. *Nat Med.* **4**, 488-92.

Mabbott NA, Bruce ME, Botto M, Walport MJ & Pepys MB. (2001). Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie. *Nat Med.* **4**, 485-7.

Nauta, A.J., Bottazzi, B., Mantovani, A., Salvatori, G., Kishore, U., Schwaeble, W.J., Gingras, A.R., Tzima, S., Vivanco, F., Egido, J., Tijsma, O., Hack. E.C., Daha, M.R. & Roos, A (2003). Biochemical and functional characterization of the interaction between pentraxin 3 and C1q. *Eur. J. Immunol.* **33**, 465-473.

Nazor, K.E., Kuhn, F., Seward, T., Green, M., Zwald, D., Purro, M., Schmid, J., Biffiger, K., Power, A. M., Oesch, B., Raeber, A. J., Telling, G. C. (2005). Immunodetection of disease-associated mutant PrP, which accelerates disease in GSS transgenic mice. *The EMBO Journal.* **24**, 2472-2480.

Outram, G.W. (1976). The pathogenesis of scrapie in mice. In *Slow virus diseases of animals and man.* Frontiers of biology series, vol 44, ch. 14, pp. 325-357. Edited by R.H. Kimberlin. Amsterdam, North Holland.

Sigurdsson, E.M., Sy, M-S., Li, R., Scholtzova, H., Kascsak, R.J., Kascsak, R., Carp, R., Meeker, H.C., Frangione, B. and Wisniewski, T. (2003). *Neuroscience Letters.* **336**, 185-187.

Schwarz, A., Kratke, O., Burwinkel, M., Riemer, C., Schultz, J., Henklein, P., Bamme, T. & Baier, M. (2003). Immunization with a synthetic prion protein-derived peptide prolongs survival times of mice orally exposed to the scrapie agent. *Neuroscience Letters.* **350**, 187-189.

Appendices

None